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(54) Title: GNRH ANTAGONISTS

(57) Abstract

Peptides having significantly modified amino acids inhibit the secretion of gonadotropins by the pituitary gland and inhibit the release of steroids by the gonads. Administration of an effective amount of such GnRH antagonists prevents ovulation of female mammalian eggs and/or the release of steroids by the gonads. These antagonists may be used to treat steroid-dependent tumors, such as prostatic and mammary tumors. A particularly effective analog of the decapeptide GnRH which is highly soluble in water at physiologic pH has the formula: Ac-β-D-2NAL-(4Cl)D-Phe-D-3PAL-Ser-N$^{\alpha}$CH$_3$Aph(3-amino 1,2,4 triazole)-D-Aph(3-amino 1,2,4 triazole)-Leu-Lys(isopropyl)-Pro-D-Ala-NH$_2$.

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GNRH ANTAGONISTS.

This invention relates generally to peptides which are antagonists of human gonadotropin releasing hormone (GnRH) and more particularly to GnRH antagonists which include significantly modified amino acids which have advantageous physical, chemical and biological properties. In a more particular aspect, the present invention relates to decapeptides which inhibit the gonadal function and the release of the steroidal hormones progesterone and testosterone, and to methods of administering such decapeptides for such purpose and particularly to prevent ovulation.

BACKGROUND OF THE INVENTION

The pituitary gland is attached by a stalk to the region in the base of the brain known as the hypothalamus. In particular, follicle stimulating hormone (FSH) and luteinizing hormone (LH), sometimes referred to as gonadotropins or gonadotropic hormones, are released by the pituitary gland. These hormones, in combination, regulate the functioning of the gonads to produce testosterone in the testes and progesterone and estrogen in the ovaries, and they also regulate the production and maturation of gametes.

The release of a hormone by the anterior lobe of the pituitary gland usually requires a prior release of another class of hormones produced by the hypothalamus. One of the hypothalamic hormones acts as a factor that triggers the release of the gonadotropic hormones, particularly LH, and this hormone is referred to herein as GnRH although it has also been referred to as LH-RH and as LRF. GnRH was isolated and characterized as a decapeptide some 20 years ago, and it was found shortly thereafter that analogs of GnRH having a D-isomer instead

of Gly in the 6-position, such as [D-Ala^6]-GnRH (U.S. Patent No. 4,072,668) having the following formula:

pGlu-His-Trp-Ser-Tyr-D-Ala-Leu-Arg-Pro-Gly-NH_2,

have greater binding strength to the receptor and greater biological potency than the native hormone.

Peptides are compounds which contain two or more amino acids in which the carboxyl group of one acid is linked to the amino group of the other acid. The formula for GnRH, as represented above, is in accordance with conventional representation of peptides where the amino terminus appears to the left and the carboxyl terminus to the right. The position of the amino acid residue is identified by numbering the amino acid residues from left to right. In the case of GnRH, the hydroxyl portion of the carboxyl group of glycine at the C-terminus has been replaced with an amino group(NH_2) i.e. the C-terminus is amidated. The abbreviations for the individual amino acid residues above are conventional and are based on the trivial name of the amino acid, e.g. pGlu is pyroglutamic acid, Glu is glutamic acid, His is histidine, Trp is tryptophan, Ser is serine, Tyr is tyrosine, Gly is glycine, Leu is leucine, Nle is norleucine, Orn is ornithine, Arg is arginine, Har is homoarginine, Pro is proline, Sar is sarcosine, Phe is phenylalanine, Ala is alanine, Val is valine, Nva is norvaline, Ile is isoleucine, Thr is threonine, Lys is lysine, Asp is aspartic acid, Asn is asparagine, Gln is glutamine, and Met is methionine. Except for glycine, amino acids of the peptides of the invention are of the L-configuration unless noted otherwise.

There are reasons for desiring to prevent ovulation in female mammalians, and the administration of GnRH analogs that are antagonistic to the normal function of GnRH have been used to suppress or delay ovulation. For this reason, analogs of GnRH which are antagonistic to GnRH are being investigated for their potential use as a

contraceptive or for regulating conception periods. GnRH antagonists may also be used for the treatment of precocious puberty and endometriosis. Such antagonists have also been found useful to regulate the secretion of gonadotropins in male mammals and can be employed to arrest spermatogenesis, e.g. as male contraceptives for treatment of male sex offenders, and for treatment of prostatic hypertrophy. More specifically, GnRH antagonists can be used to treat steroid-dependent tumors, such as prostatic and mammary tumors, and for the control of the timing of ovulation for in vitro fertilization. In the female, they can also be used to treat hirsutism.

There are a number of peptides that are known to cause histamine to be released from mast cells which cells are found in the skin, the gingiva and other locations throughout the body. As a result, inflammation is caused, often resulting in edema of the face and elsewhere on the skin. It was earlier found that certain GnRH antagonists that were effective in preventing ovulation had the undesirable adverse side effect of stimulating histamine release, generally rendering such GnRH analogs unacceptable for administration to humans. As a result, the design of GnRH analogs was directed to providing peptides that would retain the biological efficacy but would not have such undesirable histamine release, see J. Rivier et al., J. Med. Chem., 29, 1846-1851 (1986). In addition, it is felt to be important that the peptide analog should have good duration of action upon LH secretion, a property which is considered to be enhanced by resistance to proteolytic enzyme degradation in the body, and thus this property was also kept in focus in analog design. In addition to facilitate administration of these compounds to mammals, particularly humans, it is considered extremely advantageous for the GnRH decapeptide to have high

solubility in water at normal physiologic pH, i.e. about pH 5 to about pH 7.4.

In J. Rivier et al., J. Med. Chem., 35, 4270-4277 (1992), the design and synthesis of GnRH antagonists having improved properties in various of these respects are described. Despite the attractive properties of these GnRH analogs, the search has continued for still further improved GnRH antagonists.

SUMMARY OF THE INVENTION

It has now been found that GnRH antagonist decapeptides having the following formula, and closely related analogs thereof, have particularly improved properties in all of the aforementioned respects:

Ac-β-D-2NAL-(4Cl)D-Phe-D-3PAL-Ser-$N^{\alpha}CH_3$Aph(3-amino 1,2,4 triazole)-D-Aph(3-amino 1,2,4 triazole)-Leu-Lys(isopropyl)-Pro-D-Ala-NH_2.

These antagonists are particularly useful as fertility regulators in humans for they are extremely biopotent and long-acting. They exhibit negligible side effects of stimulating histamine release, and they have excellent solubility in aqueous buffers at physiologic pHs. The presence of the lower alkyl, e.g. methyl, substitution at the alpha-nitrogen of the modified phenylalanine residue in the 5-position, in combination with the side chains of the 5- and 6-position residues which include the amino triazole-substituted phenyl moieties, is believed to be responsible for the substantial improvements exhibited, including high solubility and biopotency of long-acting duration. As a result, this decapeptide finds particular use in administration to mammals, especially humans, as a fertility regulator and for the treatment of pathological conditions such as precocious puberty, hormone dependent neoplasia, dysmenorrhea, endometriosis and steroid-dependent tumors.

These GnRH antagonists are extremely soluble in the physiologic pH range of about 5 to about 7.4, and therefore they can be formulated and administered in concentrated form, particularly at a pH between about 5 and about 7. The antagonists are also effective for the contraceptive treatment of male mammals and for the treatment of steroid-dependent tumors. The antagonists are surprisingly long-acting in their suppression of LH levels following administration and have a particularly low side effect in respect of histamine release.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

U.S. Patent No. 5,169,932, issued December 8, 1992, discloses the design and synthesis of a number of GnRH antagonists wherein the side chains of selected residues are reacted to create cyanoguanidino moieties, some of which subsequently spontaneously convert to a desired heterocycle, e.g. a 3-amino, 1,2,4 triazole. The cyanoguanidino moieties are built upon the omega-amino group in an amino acid side chain such as lysine, ornithine or 4-amino phenylalanine (Aph) or an extended chain version thereof, such as 4-amino homophenylalanine (Ahp). GnRH antagonists having such significantly modified or unnatural amino acids in the 5- and 6-positions have good biological potency, and it has now been found that, in combination with a lower alkyl substitution to the alpha-amino group of the 5-position residue in the decapeptide, particularly advantageous overall properties are obtained.

Over the last decade, the particular properties of each of the 10 residues in the sequence of GnRH, from the standpoint of creating an effective antagonist, have been studied in depth, and as a result of these studies, it has been discovered that there are various equivalent residues that can be chosen and that substitutions of one of these equivalents for another does not significantly

detract from the biological potency of the decapeptide GnRH antagonist. The N-terminus is preferably N-acylated. Generally in the 1-position at the N-terminus, it has been shown that an N-acylated D-isomer having an aromatic ring structure in its side chain is suitable, such as substituted D-Phe, substituted or unsubstituted D-Trp, or β-D-Nal, and these 3 residues can generally be interchangeably employed at the N-terminus without substantially altering biological efficacy.

It has also become generally accepted that the inclusion of a para-substituted or a 2,4 chloro-disubstituted or a pentamethyl(Me_5)D-Phe residue in the 2-position adds significantly to GnRH antagonist activity and that the specific identity of the ring substituent is of only relatively minor importance when selected from among the following: chloro, fluoro, bromo, nitro, methyl and alkoxy. With respect to the 3-position of the molecule, it has been well accepted that a D-isomer having a multiple-ring side chain is preferred, and therefore D-Pal, D-Nal or D-Trp (unsubstituted or with a substitution such as $6NO_2$ or N^{in}For) can be alternatively employed without significantly changing biological antagonist activity. Although equivalents which are tolerated have also been disclosed for serine in the 4-position, such have not significantly improved biological activity, and the native residue is preferred in this position.

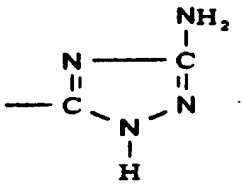
It is disclosed in U.S. Patent No. 4,652,550, issued March 24, 1987, that a number of substitutions can be made for Leu in the 7-position without significantly detracting from biological potency, and thus these potential substituents are now considered to be equivalents. However, generally Leu or $N^{\alpha}CH_3$Leu(NML) has been used, and one of these two or Nle or Phe is generally preferred. In the 8-position, the naturally-

occurring residue Arg can be employed, or it can be substituted by Har, isopropyl lysine (ILys) or isopropyl ornithine (IOrn) which are considered to be equivalents for this position. In the 9-position, the native residue Pro is preferred; however, N-methylalanine can be used as an equivalent. Although the amidated native residue Gly can be employed at the C-terminus, D-alanine having its alpha-carboxyl group amidated is preferred. Other equivalents either use AzaGly-NH_2, or omit the 10-position residue and instead use the ethylamide of proline in the 9-position.

In view of the foregoing, the present invention is broadly considered to provide a family of GnRH antagonists represented by the following formula:

G-AA_1-(A)D-Phe-AA_3-Ser-(R_1)AA_5-AA_6-AA_7-AA_8-Pro-AA_{10}

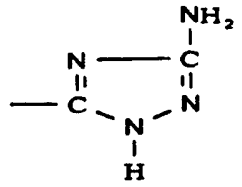
wherein G is an acyl group having 7 or less carbon atoms; AA_1 is (A)D-Phe, (B)D-Trp or β-D-NAL; A is Cl, F, NO_2, Br, CH_3, OCH_3, Me_5 or Cl_2; B is H, NO_2, OCH_3, F, Cl, Br, CH_3 or $N^{in}For$; AA_3 is D-PAL, β-D-NAL or (B)D-Trp; AA_5 is Xaa; R_1 is $N^{\alpha}CH_3$ or $N^{\alpha}CH_2CH_3$; AA_6 is D-Xaa; AA_7 is Leu, NML, Nle, Phe, Met, Nva, Tyr, Trp or PAL; AA_8 is Arg, Har, ILys or IOrn; AA_{10} is D-Ala-NH_2, Gly-NH_2, AzaGly-NH_2 or NH(R_2); R_2 is lower alkyl, preferably CH_2CH_3; and Xaa is Aph, Ahp, Lys or Orn having its ω-amino group substituted by



By β-D-NAL is meant the D-isomer of alanine which is substituted by naphthyl on the β-carbon atom, i.e., also 3-D-NAL. Preferably β-D-2NAL is employed wherein the attachment to naphthalene is at the 2-position on the ring structure; however, β-D-1NAL may also be used. The

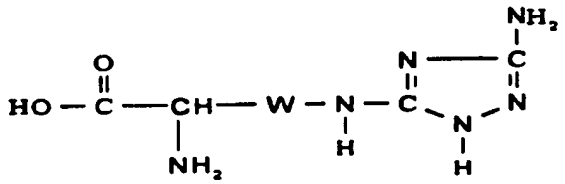
preferred 1-position residues are β-D-NAL, substituted D-Phe and optionally substituted D-Trp. PAL represents alanine which is substituted by pyridyl on the β-carbon atom; preferably the linkage is to the 3-position on the pyridine ring. When substituted D-Trp is employed, single substitutions for hydrogen are preferably made in either the 5- or 6-position, which are selected from chloro, fluoro, bromo, methyl, amino, methoxy and nitro, with chloro, fluoro and nitro being preferred. Alternatively, the indole nitrogen may be acylated with formyl (N^{in}For- or 1For-). D-3PAL, N^{in}For-D-Trp and $6NO_2$-D-Trp are the preferred residues for the 3-position although β-D-2Nal and D-Trp are also often used. By dehydroPro is meant 3,4 dehydroproline, $C_5H_7O_2N$ (Δ^3Pro). By NML is meant $N^{\alpha}CH_3$-L-Leu. By Aph is meant $4NH_2$Phe; by Ahp is meant $4NH_2$-homophenylalanine. By AzaGly-NH_2 is meant $NHNHCONH_2$. The 7-position residue is preferably Leu, NML, Nle or Phe, and the 8-position residue is preferably ILys.

A preferred subgenus of GnRH antagonists has the formula:
Ac-β-D-2NAL-(4Cl)D-Phe-D-3PAL-Ser-(R_1)AA_5-AA_6-AA_7-Lys(isopropyl)-Pro-AA_{10} wherein AA_5 is Xaa, R_1 is $N^{\alpha}CH_3$ or $N^{\alpha}CH_2CH_3$, AA_6 is D-Xaa, AA_7 is Leu or $N^{\alpha}CH_3$Leu; AA_{10} is D-Ala-NH_2, Gly-NH_2, or $NHCH_2CH_3$; Xaa is Aph(atz), Ahp(atz), Lys(atz) or Orn(atz); and atz is



An additional preferred subgenus of GnRH antagonists has the formula:
Ac-β-D-2NAL-(4Cl)D-Phe-D-3PAL-Ser-(R_1)AA_5-AA_6-AA_7-Lys(isopropyl)-Pro-AA_{10} wherein AA_5 is Xaa; R_1 is $N^{\alpha}CH_3$ or

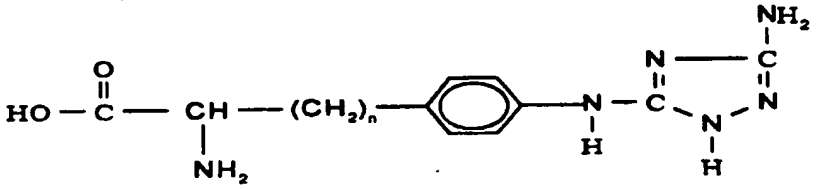
$N^{\alpha}CH_2CH_3$; AA_6 is D-Xaa; AA_7 is Leu or NML; AA_{10} is D-Ala-NH_2, Gly-NH_2 or $NHCH_2CH_3$; and Xaa is



where W is $(CH_2)_n$  ; n is 1 or 2.

Another preferred subgenus of GnRH antagonists has the formula:

Ac-β-D-2NAL-(4Cl)D-Phe-D-3PAL-Ser-$N^{\alpha}CH_3AA_5$-AA_6-Leu-Lys(isopropyl)-Pro-D-Ala-NH_2, wherein AA_5 is Xaa; AA_6 is D-Xaa and Xaa is



wherein n is 1 or 2 and preferably is 1.

The peptides of the present invention can be synthesized by classical solution synthesis, but are preferably synthesized by a solid phase technique. A chloromethylated resin or a hydroxymethylated resin may be used; however, a methylbenzhydrylamine(MBHA) resin, a benzhydrylamine (BHA) resin or some other suitable resin known in the art which directly provides a C-terminal amide or substituted amide upon cleavage is preferably employed. For example, peptides having a substituted amide at the C-terminus are preferably synthesized using an N-alkylamino methyl resin as taught in United States

Patent No. 4,569,967, issued February 11, 1986. Solid phase synthesis is conducted in a manner to stepwise add amino acids in the chain in the manner set forth in detail in the U.S. Patent No. 4,211,693. Side-chain protecting groups, as are well known in the art, are preferably included as a part of any amino acid which has a particularly reactive side chain and optionally in the case of others, such as Trp, which amino acids are to be coupled in the chain being built upon the resin. Such synthesis provides the fully protected intermediate peptidoresin.

Chemical intermediates made generally in accordance with the invention may be represented by the formula: X^1-$AA_1(X^2)$-(A)D-Phe-$AA_3(X^2)$-Ser(X^3)-$(R_1)AA_5$-AA_6-$AA_7(X^2$ or $X^4)$-$AA_8(X^5$ or $X^6)$-Pro-X^7 wherein X^1 is an α-amino protecting group of the type known to be useful in the art in the stepwise synthesis of polypeptides and when G in the desired peptide composition is a particular acyl group, that group may be used as the protecting group. Among the classes of α-amino protecting groups covered by X^1 are (1) acyl-type protecting groups, such as formyl(For), trifluoroacetyl, phthalyl, p-toluene-sulfonyl(Tos), benzoyl(Bz), benzenesulfonyl, dithiasuccinoyl(Dts) o-nitrophenylsulfenyl(Nps), tritylsulfenyl, o-nitrophenoxyacetyl, acrylyl(Acr), chloroacetyl, acetyl(Ac) and γ-chlorobutyryl; (2) aromatic urethan-type protecting groups, e.g., benzyloxycarbonyl(Z), fluorenylmethyloxycarbonyl(Fmoc) and substituted benzyloxycarbonyl, such as p-chlorobenzyloxy-carbonyl(ClZ), p-nitrobenzyloxycarbonyl, p-bromobenzyloxycarbonyl and p-methoxybenzyloxycarbonyl; (3) aliphatic urethan protecting groups, such as tertbutyloxycarbonyl(Boc), diisopropylmethoxycarbonyl, isopropyloxycarbonyl, ethoxycarbonyl and allyloxycarbonyl; (4) cycloalkyl

urethan-type protecting groups, such as cyclopentyloxycarbonyl, adamantyloxycarbonyl and cyclohexyloxycarbonyl; (5) thiourethan-type protecting groups, such as phenylthiocarbonyl; (6) alkyl-type protecting groups, such as allyl(Aly), triphenylmethyl(trityl) and benzyl(Bzl); (7) trialkylsilane groups, such as trimethylsilane. The preferred α-amino protecting group is Boc when X is hydrogen.

X^2 is hydrogen or a protecting group for the indole nitrogen of Trp, such as Bz, Ac or For. In many syntheses there is no need to protect Trp, and such protection is not used if acylated D-Trp is present elsewhere in the peptide.

X^3 is a protecting group for the hydroxyl side chain of Ser, e.g. Ac, Bz, trityl, DCB or benzyl ether(Bzl) and is preferably Bzl.

X^4 is hydrogen or a protecting group for the phenolic hydroxyl group of Tyr selected from the group consisting of tetrahydropyranyl, tert-butyl, trityl, benzyl, Z, 2-bromobenzyloxycarbonyl(2BrZ) and 2,6-dichloro-benzyl(DCB). 2BrZ is preferred.

Met, if used, may be protected by oxygen, but Met is generally left unprotected.

X^5 is a protecting group for a side chain guanidino group in Arg or Har, such as nitro, Tos, trityl, adamantyloxycarbonyl, Z and 2,4-dinitrophenol(Dnp), or X^5 may be hydrogen, which means there is no protection on the side chain group atoms. Tos is generally preferred.

X^6 is a protecting group for an amino side chain group, primary or secondary amino, such as Z or 2ClZ.

X^7 may be Gly-NH-[resin support], D-Ala-NH-[resin support] or N(A)-[resin support]; X^7 may also be an amide either of Gly or of D-Ala or a substituted amide attached directly to Pro or $NHNHCONH_2$.

The criterion for selecting certain side chain protecting groups for X^2-X^6 is that the protecting group should be stable to the reagent under the reaction conditions selected for removing the α-amino protecting group (preferably Boc) at each step of the synthesis. These protecting groups generally should not be split off under coupling conditions but should be removable upon completion of the synthesis of the desired amino acid sequence under reaction conditions that will not alter the peptide chain. Other protecting groups initially employed for the 5- and 6-position residues are removed prior to cleavage from the resin, as explained hereinafter.

When the X^7 group is Gly-NH-[resin support] or D-Ala-NH-[resin support], an amide bond connects Gly or D-Ala to a BHA resin or to a MBHA resin. When the X^7 group is N(A)-[resin support], a substituted amide bond connects Pro to an N-alkylaminomethyl (NAAM) resin. When X^7 is AzaGly-NH_2, the peptide is preferably made by classical solution synthesis, as disclosed in U.S. Patent No. 4,234,571.

When G is acetyl, for example, in the final formula, it may be possible to employ it as the X^1 protecting group for the α-amino group of β-D-NAL or whatever amino acid is used in the 1-position by adding it before coupling this last amino acid to the peptide chain. However, a reaction is preferably carried out with the peptide on the resin (after deblocking the α-amino group while the side-chain groups remain protected), e.g. by reacting with acetic acid, or preferably with acetic anhydride, in the presence of diisopropyl or dicyclohexyl carbodiimide (DIC or DCC) or by some other suitable reaction as known in the art.

Thus, the invention also provides a method for making a GnRH peptide having the formula:

G-AA_1-(A)D-Phe-AA_3-Ser-(R_1)AA_5-AA_6-AA_7-AA_8-Pro-AA_{10}, wherein AA_1, AA_3, AA_5, AA_6, AA_7, AA_8 and AA_{10} are as set forth hereinbefore, which method comprises (a) forming an intermediate peptide having the formula: X^1-AA_1(X^2)-(A)D-Phe-AA_3(X^2)-Ser(X^3)-(R_1)AA_5(X^a)-AA_6(X^a)-AA_7(X^2 or X^4)-AA_8(X^5 or X^6)-Pro-X^7 wherein X^1 is hydrogen or an α-amino protecting group; X^2 is hydrogen or a protecting group for an indole nitrogen; X^3 is a protecting group for a hydroxyl group of Ser; X^4 is hydrogen or a protecting group for a phenolic hydroxyl group of Tyr; X^5 is either hydrogen or a protecting group for a guanidino side chain; X^6 is a protecting group for an amino side chain, of which X^a is a subgroup that is removable without removing other protecting groups; X^7 is Gly-NH-[resin support], D-Ala-NH-[resin support], N(A)-[resin support], an amide either of Gly or of D-Ala or a substituted amide attached directly to Pro or $NHNHCONH_2$; (b) removing X^a from AA_5 and AA_6 to deprotect a side chain primary amino group of these amino acid residues of said intermediate peptide; (c) reacting said deprotected side chain primary amino groups to build said residue into one having an animo-triazole moiety; and (d) splitting off any remaining groups X^1 to X^6 and/or cleaving from any resin support included in X^7.

Purification of the peptide is effected by ion exchange chromatography on a CMC column, followed by partition chromatography using the elution system: n-butanol;0.1N acetic acid (1:1 volume ratio) on a column packed with Sephadex G-25, or by using HPLC, as known in the art and specifically set forth in J. Rivier, et al. J. Chromatography, 288 (1984) 303-328.

The antagonists of the invention are effective at levels of less than 100 micrograms per kilogram of body

weight, when administered subcutaneously at about noon on the day of proestrus, to prevent ovulation in female rats. For prolonged suppression of ovulation, it may be necessary to use dosage levels in the range of from about 0.1 to about 2.5 milligrams per kilogram of body weight. These analogs are particularly soluble at physiological pHs and thus can be prepared as relatively concentrated solutions for administration. The antagonists are also effective to arrest spermatogenesis when administered to male mammals on a regular basis and can thus be used as contraceptives. Since these compounds will reduce testosterone levels (an undesired consequence in the normal, sexually active male), it may be desirable to administer replacement dosages of testosterone along with the GnRH antagonist. These antagonists can also be used to regulate the production of gonadotropins and sex steroids for other purposes as indicated hereinbefore.

In the following formulas, the residues for positions 5 and 6 are sometimes defined in terms of the original amino acid residue having a side chain amino group plus the modification in question which is set forth in the accompanying parentheses. Preferably, the original residue is incorporated in the main peptide chain, for example Aph, Ahp, Lys, Orn or the respective D-isomer thereof, and is modified while a part of the peptide chain that is still attached to the resin to form the desired residue AA_5 or AA_6. However, the suitably protected significantly modified amino acid can be added to the growing peptide chain as a part of the usual chain elongation process, if desired.

The following Examples illustrate a number of GnRH antagonist peptides embodying various features of the invention. All of these peptides include at least one D-isomer amino acid residue. The N-alkyl modification of the amino acid to be included in the 5-position can be

made prior to its linkage to the peptidoresin (as described below) or the α-amino group can be alkylated after the residue has been attached to the peptidoresin during the solid-phase synthesis using steps known in the art.

EXAMPLE 1

Preparation Of N^{α}Boc-N^{α}methyl(4-Fmoc-amino)L-phenylanaline

40 grams of Boc-L-Phe(0.152 mole) were dissolved in 100 milliliters of tetrahydrofuran (THF) and added dropwise to 13.58 grams of NaH(0.452 mole) (3eq) which had been dissolved in 500 milliliters of THF at 0°C. Thereafter, 42.6 grams of methyl iodide (2eq), dissolved in THF, were added dropwise over 30 minutes, and stirring in a beaker was continued for about 36 hours at room temperature. The reaction was stopped by placing the beaker in an ice bath and by the slow addition of 20 milliliters of glacial acetic acid and 20 milliliters of distilled water. The THF was removed by rotary evaporation, and the remaining oil was diluted with 300 milliliters of water. After adjusting to a pH of about 8 with sodium carbonate, the mixture was extracted twice, using 200 milliliters of ethyl ether each time. Following extraction, the remainder is acidified to a pH of about 2, with sulfuric acid, and it is then extracted twice with 300 milliliters of ethylacetate. After drying over anhydrous magnesium sulfate, solvent removal is effected by evaporation, giving about 40 grams of an oil which crystallizes. The crystals of Boc-N^{α}MePhe have a melting point of 71°C and, when dissolved in CH_3OH, have an $[\alpha]_D$ equal to -93.6 ±1(c = 1.1, methanol).

31 grams of this Boc-protected amino acid (0.11 mole) were cooled to 0°C and stirred together with 30 milliliters of trifluoroacetic acid (TFA) and 1

milliliter of anisole for 30 minutes at 0°C. The mixture was then diluted with 600 milliliters of t-butylmethyl ether and 1.4 liters of ethyl ether to precipitate the amino acid, which is collected by filtering. The amino acid is then dissolved in 50 milliliters of water, and the pH is adjusted to about 6 with ammonium hydroxide. Drying overnight over high vacuum produced 12 grams of N^{α}MePhe having a melting point of about 253°C and $[\alpha]_D$ = +15.4° ±1(c = 0.96, 1N HCl).

The N-alpha-methyl-Phe is then substituted with nitrate in the 4- or para-position as follows. A mixture of 40 milliliters of 95% sulfuric acid and 4 milliliters of 70% nitric acid was cooled to about -2°C, and 11.6 grams of the amino acid (0.065 mole) were added slowly over 30 minutes with stirring, which was continued for about one hour. The mixture was then poured onto 350 grams of ice, and the pH was adjusted to about 6 with ammonium hydroxide. The amino acid was recrystallized three times from water at 90°C to assure purification. Then drying was carried out under high vacuum, which produced 8.7 grams (0.039 mole) of N^{α}Me4NO_2Phe having a melting point of about 255°C and an $[\alpha]_D$ = +32.8° ±1(c = 1, 1N HCl). 8.5 grams of the N^{α}Me4NO_2Phe (0.036 mole) were reacted with Boc-carbonate under standard conditions to produce (Boc-N^{α}Me4NO_2Phe in an amount of about 11.5 grams and in the form of a yellow oil, which crystallizes and has an $[\alpha]_D$ = -73.6° ± 1(c = 0.8, 50% acetic acid).

This compound was then reduced using a palladium catalyst on a carbon substrate in a mixture of equal parts of ethanol and acetic acid by bubbling hydrogen gas through the mixture until uptake of H_2 ceased. The solvent was then removed by rotary evaporation, and the remaining compound was dissolved in water and its pH adjusted to about 3. Extraction was twice carried out with 50 milliliters of ethyl ether and then twice with 50

milliliter portions of ethylacetate. The pH is then adjusted to 9.4 with ammonium hydroxide.

The side chain amino group is next protected with Fmoc by reaction with 9-fluorenylmethyl-succinimidylcarbonate (Fmoc-Osu) under standard conditions. The resultant Boc-N^{α}Me4NH_2Phe(Fmoc) having an $[\alpha]_D$ = -43.8° ±1(c = 1.2, ethylacetate). This N^{α}-Boc-protected and Fmoc-side chain-protected Phe is then used in the peptide synthesis which follows.

EXAMPLE 2

Peptide No. 1 in the form of the decapeptide [Ac-β-D-2NAL[1], (4Cl)D-Phe[2], D-3PAL[3], N^{α}MeAph(atz)[5], D-Aph(atz)[6], ILys[8], D-Ala[10]]-GnRH is synthesized by solid-phase synthesis. This peptide has the following formula: [Ac-β-D-2NAL-(4Cl)D-Phe-D-3PAL-Ser-$N^{\alpha}CH_3$Aph(3-amino 1,2,4 triazole)-D-Aph(3-amino 1,2,4 triazole)-Leu-Lys(isopropyl)-Pro-D-Ala-NH_2.

About 1.5 grams (0.76 mM/g) of MBHA resin are used, and Boc-protected D-Ala is coupled to the resin over a 2-hour period in CH_2Cl_2 using about 2 millimoles of Boc derivative and DCC as an activating reagent. The D-Ala residue attaches to the MBHA residue by an amide bond.

Following the coupling of each amino acid residue, washing, deblocking and coupling of the next amino acid residue is carried out in accordance with the following schedule using an automated machine and beginning with about 1.5 grams of resin:

STEP	REAGENTS AND OPERATIONS	MIX TIMES MIN.
1	CH_2Cl_2 wash-80 ml. (2 times)	3
2	Methanol(MeOH) wash-30 ml. (2 times)	3
3	CH_2Cl_2 wash-80 ml. (3 times)	3
4	50 percent TFA plus 5 percent 1,2-ethanedithiol in CH_2Cl_2-70 ml. (2 times)	10
5	Isopropyl alcohol + 1% ethanedithiol wash-80 ml. (2 times)	3
6	TEA 12.5 percent in CH_2Cl_2-70 ml.	5
7	MeOH wash-40 ml. (2 times)	2
8	TEA 12.5 percent in CH_2Cl_2-70 ml.	5
9	CH_2Cl_2 wash-80 ml. (3 times)	3
10	Boc-amino acid (10 mmoles) in 30 ml. of either dimethylformamide(DMF): CH_2Cl_2 or DMF alone, depending upon the solubility of the particular protected amino acid, plus DIC or DCC (10 mmoles) in CH_2Cl_2	30-300
11	MeOH wash-40 ml. (2 times)	3
12	Triethylamine(TEA) 12.5 percent in CH_2Cl_2-70 ml.	3
13	MeOH wash-30ml. (2 times)	3
14	DCM wash-80 ml. (2 times)	3

The above schedule is used for coupling of each of the amino acids of the peptide of the invention after the first amino acid has been attached. N^{α}Boc protection is used for each of the remaining amino acids throughout the synthesis. N^{α}Boc-β-D-2NAL is prepared by a method known in the art, e.g. as described in detail in U.S. Patent No. 4,234,571, issued November 18, 1980; it is also commercially available from SyntheTech, Oregon, U.S.A. The side chain primary amino groups of N^{α}MeAph in the 5-

position and of D-Aph in the 6-position are protected by Fmoc, with the amino acid for the 5-position being prepared by the process set forth in Example 1. Bzl(benzyl ether) is used as a side chain protecting group for the hydroxyl group of Ser. Boc-Lys(Ipr,Z) is used for the 8-position. After deblocking the α-amino group at the N-terminus using trifluoroacetic acid (TFA), acetylation is achieved using a large excess of acetic anhydride in dichloromethane.

Following completion of the assembly of the peptide and acetylation of the N-terminus, about 3.2 grams of the following intermediate are present: Ac-β-D-2NAL-(4Cl)D-Phe-D-3PAL-Ser(Bzl)-$N^{\alpha}CH_3$Aph(Fmoc)-D-Aph(Fmoc)-Leu-Lys(Ipr,Z)-Pro-D-Ala-NH-[MBHA resin support]. The side chains on the amino acid residues in the 5- and 6-positions are modified by simultaneously carrying out the following reactions with the deprotected side chains of the Aph residues. The Fmoc protecting group is removed from both residues by treatment of the peptidoresin with 20 percent piperidine in DMF for about 15 minutes; it is preferably washed with DMF and then treated with more piperidine/DMF for 15 minutes. After preferably washing the peptidoresin with DMF, the newly freed amino groups are treated with a large excess (>10 fold) of diphenyl cyanocarbonimidate(PCI) in 5 ml. of DMF and left to react overnight at room temperature. Thereafter, the peptide is washed with DMF, CH_3OH and CH_2Cl_2, and this standard wash is repeated. Then, the peptidoresin is treated with 5 ml. of hydrazine dissolved in 5 ml. of DMF, for 12 hours at about 22°C. to complete the formation of the cyanoguanidino moiety; this step is preferably repeated. The cyanoguanidino moieties that are formed spontaneously convert to the corresponding heterocycle, i.e. 3-amino, 1,2,4 triazole. The standard wash is then carried out and repeated.

The peptidoresin is dried, and then cleavage of the peptide from the resin and deprotection of the Ser and the Lys side chains is carried out at 0°C. with HF. Anisole is added as a scavenger prior to HF treatment. After the removal of HF under vacuum, the resin is washed twice with 100 ml. of ethyl ether. The cleaved peptide is extracted from the resin with equal parts of CH_3CN and H_2O, repeating the process and using 150 ml. each time. The extracts are pooled and lyophilized, and they provide about 762 mg of a crude peptide powder.

Purification of the peptide is then effected by preparative high performance liquid chromatography (HPLC), as known in the art and specifically set forth in J. Rivier, et al. J. Chromatography, 288, 303-328 (1984). The first preparative RP-HPLC separation uses a TEAP (triethylammonium phosphate) buffer system. This separation is repeated using the same buffer system with a slightly different gradient, and the final separation is carried out using a 0.1% TFA (trifluoroacetic acid) gradient, all as described in detail in the J. Chromatography article. About 204 milligrams of the decapeptide are obtained.

The peptide is judged to be homogeneous using capillary zone electrophoresis (CZE), as well as by using reversed-phase high pressure liquid chromatography and an aqueous triethylammonium phosphate buffer plus acetonitrile. The purity is estimated to be about 99.2%. Amino acid analysis of the resultant, purified peptide is consistent with the formula for the prepared structure, showing substantially integer-values for each amino acid in the chain; mass spectral analysis is also consistent. The optical rotation is measured on a photoelectric polarimeter as

$[\alpha]_D^{20} = -43.1° \pm 1.0$(c=0.63, 50% acetic acid).

The peptide is assayed in vivo to determine its effectiveness to prevent ovulation in female rats. In this test, a specified number of mature female Sprague-Dawley rats, e.g. five to ten, each having a body weight from 225 to 250 grams, are injected with a specified microgram dosage of the peptide in saline, bacteriostatic water, polyethylene glycol, corn oil or a mixture of one of the above with ethanol, at about noon on the day of proestrus. Proestrus is the afternoon of ovulation. A separate female rat group is used as a control to which the peptide is not administered. Each of the control female rats ovulates on the evening of proestrus; the number of the rats treated which ovulate is recorded. In vivo testing of the peptide shows that, at a dosage of 2.5 micrograms, 0 out of 8 rats treated ovulate, and at a dosage of 1 microgram, only 2 out of 9 rats ovulate.

In vitro testing is carried out using dissociated rat pituitary cells maintained in culture for 4 days prior to the assay. The levels of LH mediated in response to the application of peptides is assayed by specific radioimmunoassay for rat LH. Control dishes of cells only receive a measure which is 3 nanomolar in GnRH; experimental dishes receive a measure 3 nanomolar in GnRH plus a measure having either the present standard antagonist for comparison purposes i.e. [Ac-dehydro Pro^1, (4F)D-Phe^2, D-$Trp^{3,6}$]-GnRH or the test peptide, in concentrations ranging from 0.01 to 10 nanomolar. The amount of LH secreted in the samples treated only with GnRH is compared with that secreted by the samples treated with the peptide plus GnRH. The peptide is twice as potent as our standard [Ac-Δ^3Pro^1, D-4FPhe^2, D-$Trp^{3,6}$]-GnRH.

The peptide is considered to be particularly useful because of its substantially greater solubility in

aqueous buffers at a pH of from about 5 to about 7, compared to other compounds of generally comparable biological efficacy. This solubility is believed to be due to the fact that the lower alkyl, e.g. CH_3, group on the α-nitrogen of the amino acid in position 5 breaks the hydrogen bond responsible for β-sheet formation in combination with the effect provided by the particular side-chains on the 5- and 6-position residues which incorporate triazole-substituted phenylalanine moieties.

EXAMPLE 3

The following peptides indicated in TABLE A, having the general formula: Ac-β-D-2NAL-(4Cl)D-Phe-D-3PAL-Ser-($N^{\alpha}R_1$)AA_5-AA_6-Leu-AA_8-Pro-D-Ala-NH_2 are prepared by the solid-phase procedure generally referred to above.

TABLE A

No.	R_1	AA_5	AA_6	AA_8
2	Et	Aph(atz)	D-Aph(atz)	ILys
3	Me	Aph(atz)	D-Lys(atz)	Arg
4	Et	Orn(atz)	D-Lys(atz)	Arg
5	Me	Lys(atz)	D-Lys(atz)	ILys
6	Me	Orn(atz)	D-Orn(atz)	Har
7	Et	Lys(atz)	D-Lys(atz)	ILys
8	Me	Aph(atz)	D-Aph(atz)	IOrn
9	Me	Aph(atz)	D-Aph(atz)	Arg
10	Me	Lys(atz)	D-Lys(atz)	IOrn
11	Me	Lys(atz)	D-Ahp(atz)	Har
12	Me	Lys(atz)	D-Lys(atz)	Arg
13	Et	Orn(atz)	D-Aph(atz)	Arg
14	Me	Lys(atz)	D-Lys(atz)	Har

By atz is meant 3-amino, 1,2,4 triazole.

The peptides listed in Table A are considered effective to block GnRH-induced LH secretion in vitro at a reasonable concentration. All of the peptides are considered to be effective to prevent ovulation of female mammals at low dosages.

EXAMPLE 4

Peptides as indicated in TABLE B having the formula: G-AA_1-(A)D-Phe-AA_3-Ser-$N^{\alpha}CH_3$Aph(atz)-D-Aph(atz)-AA_7-ILys-Pro-AA_{10} are prepared by the solid-phase procedure generally referred to above.

TABLE B

No.	G	AA_1	A	AA_3	AA_7	AA_{10}
15	Ac	D-Trp	4F	D-Trp	Leu	D-Ala-NH_2
16	Acr	6NO_2D-Trp	4Cl	βD-2NAL	NML	Gly-NH_2
17	For	Me_5D-Trp	4Br	6FD-Trp	NML	$NHCH_2CH_3$
18	Bz	4FD-Phe	4NO_2	ForD-Trp	Leu	Gly-NH_2
19	Acr	βD-1NAL	4CH_3	6NO_2D-Trp	NML	AzaGly-NH_2
20	Ac	4ClD-Phe	4F	βD-1NAL	Nle	D-Ala-NH_2
21	Bz	βD-2NAL	4OCH_3	D-3PAL	Trp	Gly-NH_2
22	Acr	4NO_2D-Phe	4F	βD-1NAL	Phe	D-Ala-NH_2
23	Ac	βD-2NAL	2,4Cl_2	6BrD-Trp	Nva	$NHCH_2CH_3$
24	For	4CH_3D-Phe	4F	6CH_3D-Trp	Nle	Gly-NH_2
25	Ac	6ClD-Trp	4Br	6ClD-Trp	3PAL	D-Ala-NH_2
26	Acr	6FD-Trp	4F	D-2PAL	Met	AzaGly-NH_2

The peptides listed in Table B are considered effective to block GnRH-induced LH secretion in vitro at a reasonable concentration. All of the peptides are considered to be effective to prevent ovulation of female mammals at low dosages.

The peptides of the invention are often administered in the form of pharmaceutically acceptable, nontoxic salts, such as acid addition salts, or of metal

complexes, e.g., with zinc, barium, calcium, magnesium, aluminum or the like (which are considered as addition salts for purposes of this application), or of combinations of the two. Illustrative of such acid addition salts are hydrochloride, hydrobromide, sulphate, phosphate, nitrate, oxalate, fumarate, gluconate, tannate, maleate, acetate, citrate, benzoate, succinate, alginate, malate, ascorbate, tartrate and the like. For example, an aqueous solution of the peptide can be repeatedly treated with 1N acetic acid and then lyophilized to yield the acetic acid salt thereof. If the active ingredient is to be administered in tablet form, the tablet may contain a pharmaceutically-acceptable diluent which includes a binder, such as tragacanth, corn starch or gelatin; a disintegrating agent, such as alginic acid; and a lubricant, such as magnesium stearate. If administration in liquid form is desired, sweetening and/or flavoring may be used as part of the pharmaceutically-acceptable diluent, and intravenous administration in isotonic saline, phosphate buffer solutions or the like may be effected.

The pharmaceutical compositions will usually contain the peptide in conjunction with a conventional, pharmaceutically-acceptable carrier. Usually, the dosage will be from about 10 micrograms to about 2.5 milligrams of the peptide per kilogram of the body weight of the host when given intravenously. Oral dosages would be higher; however, the nature of these compounds should permit effective oral administration. Overall, treatment of subjects with these peptides is generally carried out in the same manner as the clinical treatment using other antagonists of GnRH using a suitable carrier in which the peptide is soluble.

It may also be desirable to deliver the GnRH analog over prolonged periods of time, for example, for periods

of one week to one year from a single administration, and slow release, depot or implant dosage forms may be utilized. For example, a suitable, slow-release depot formulation for injection may contain the GnRH antagonist or a salt thereof dispersed or encapsulated in a slow degrading, non-toxic or non-antigenic polymer such as a polylactic acid/polyglycolic acid polymer, for example, as described in U.S. Pat. No. 3,773,919. These compounds may also be formulated into silastic implants.

These peptides can be administered to mammals intravenously, subcutaneously, intramuscularly, orally, percutaneously, e.g. intranasally or intravaginally to achieve fertility inhibition and/or control and also in applications calling for reversible suppression of gonadal activity, such as for the management of precocious puberty or during radiation- or chemotherapy. They are also useful for treatment of steroid-dependent tumors. Effective dosages will vary with the form of administration and the particular species of mammal being treated. An example of one typical dosage form is a bacteriostatic water solution at a pH of about 6 containing the peptide which solution is administered parenterally to provide a dose in the range of about 0.1 to 2.5 mg/kg of body weight per day.

Although the invention has been described with regard to its preferred embodiments, it should be understood that changes and modifications as would be obvious to one having the ordinary skill in this art may be made without departing from the scope of the invention which is set forth in the claims which are appended hereto. For example, other substitutions known in the art which do not significantly detract from the effectiveness of the peptides may be employed in the peptides of the invention. D-2PAL and D-4PAL are

considered to be equivalents of D-3PAL. Other equivalent acylating groups can be used instead of acetyl at the N-terminus. Substituted Phe, such as (4F)Phe, can be used instead of Phe in the 7-position. Har is considered the equivalent of Arg in the 8-position, and both butyl Lys and diethyl Lys are considered to be equivalents of ILys. However, ILys is most preferred. Other hydrophobic amino acid residues can also be employed in the 1-position, preferably in D-isomer form, and are considered equivalents of those specified. Moreover, the antagonists can be administered in the form of their pharmaceutically or vetinarially acceptable, nontoxic salts, as indicated hereinbefore, which are considered equivalents.

Particular features of the invention are emphasized in the claims which follow.

WHAT IS CLAIMED IS:

1. A GnRH antagonist peptide, or a nontoxic salt thereof, having the formula: Ac-β-D-2NAL-(4Cl)D-Phe-D-3PAL-Ser-$N^{\alpha}CH_3AA_5$(3-amino 1,2,4 triazole)-AA_6(3-amino 1,2,4 triazole)-Leu-Lys(isopropyl)-Pro-D-Ala-NH_2, wherein AA_5 is Aph or Lys and AA_6 is D-Aph or D-Lys.

2. A GnRH antagonist peptide, or a nontoxic salt thereof, according to Claim 1 having the formula: Ac-β-D-2NAL-(4Cl)D-Phe-D-3PAL-Ser-$N^{\alpha}CH_3$Aph(3-amino 1,2,4 triazole)-D-Aph(3-amino 1,2,4 triazole)-Leu-Lys(isopropyl)-Pro-D-Ala-NH_2.

3. A GnRH antagonist peptide, or a nontoxic salt thereof, according to Claim 1 having the formula: Ac-β-D-2NAL-(4Cl)D-Phe-D-3PAL-Ser-$N^{\alpha}CH_3$Lys(3-amino 1,2,4 triazole)-D-Lys(3-amino 1,2,4 triazole)-Leu-Lys(isopropyl)-Pro-D-Ala-NH_2.

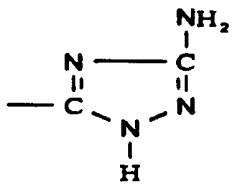
4. A method for inhibiting the secretion of gonadotropins in mammals comprising administering to a mammal an effective amount of a GnRH antagonist peptide or a nontoxic salt thereof as defined in Claim 1.

5. A GnRH antagonist peptide or a nontoxic salt thereof, said peptide having the formula:

G-AA_1-(A)D-Phe-AA_3-Ser-(R_1)AA_5-AA_6-AA_7-AA_8-Pro-AA_{10}

wherein G is an acyl group having 7 or less carbon atoms; AA_1 is (A)D-Phe, (B)D-Trp or β-D-NAL; A is Cl, F, NO_2, Br, CH_3, OCH_3, Me_5 or Cl_2; B is H, NO_2, OCH_3, F, Cl, Br, CH_3 or N^{in}For; AA_3 is D-PAL, β-D-NAL or (B)D-Trp; AA_5 is Xaa; R_1 is $N^{\alpha}CH_3$ or $N^{\alpha}CH_2CH_3$; AA_6 is D-Xaa; AA_7 is Leu, NML, Nle, Phe, Met, Nva, Tyr, Trp or PAL; AA_8 is ILys, Arg, Har, or

IOrn; AA_{10} is D-Ala-NH_2, Gly-NH_2, AzaGly-NH_2 or $NH(R_2)$; R_2 is lower alkyl; and Xaa is Aph, Ahp, Lys or Orn having its ω-amino group substituted by



6. A GnRH antagonist peptide according to Claim 5 wherein AA_1 is β-D-2NAL.

7. A GnRH antagonist peptide according to Claim 5 wherein AA_3 is D-3PAL.

8. A GnRH antagonist peptide according to Claim 5 wherein AA_3 is $6NO_2$-D-Trp.

9. A GnRH antagonist peptide according to Claim 5 wherein AA_5 is Aph(atz).

10. A GnRH antagonist peptide according to Claim 9 wherein R_1 is $N^{\alpha}CH_3$.

11. A GnRH antagonist peptide according to Claim 10 wherein AA_6 is D-Aph(atz).

12. A GnRH antagonist peptide according to Claim 11 wherein AA_7 is Leu, NML, Nle or Phe.

13. A GnRH antagonist peptide according to Claim 12 wherein AA_8 is ILys or Arg.

14. A GnRH antagonist peptide according to Claim 13 wherein AA_{10} is D-Ala-NH_2.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 94/01891

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C07K7/06 A61K37/43

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,91 06543 (THE SALK INSTITUTE FOR BIOLOGICAL STUDIES) 16 May 1991 cited in the application see the whole document ---	1-14
A	WO,A,92 01725 (THE SALK INSTITUTE FOR BIOLOGICAL STUDIES) 1 October 1992 see the whole document ---	1-14
A	JOURNAL OF MEDICINAL CHEMISTRY. vol. 35, no. 23 , 1992 , WASHINGTON US pages 4270 - 4278 J.RIVIER ET AL 'GnRH antagonists with N-omega-triazolylornithine, -lysine or -p-aminophenylalanine residues at positions 5 and 6' cited in the application see the whole document ---	1-14

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 June 1994	30. 06. 94

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Groenendijk, M

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 94/01891

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP,A,0 413 209 (ABBOTT LABORATORIES) 20 February 1991 see the whole document	1-14

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/01891

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark : Although claim 4 is directed to a method of treatment of (diagnostic method practised on) the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/US 94/01891

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9106543	16-05-91	US-A-	5169932	08-12-92
		AU-B-	633384	28-01-93
		AU-A-	6739290	31-05-91
		CA-A-	2066184	01-05-91
		EP-A-	0500695	02-09-92
		JP-T-	5503689	17-06-93
		US-A-	5296468	22-03-94
WO-A-9201725	06-02-92	DE-A-	4023005	23-01-92
		EP-A-	0539406	05-05-93
		JP-T-	5509114	16-12-93
		TR-A-	25140	01-11-92
EP-A-0413209	20-02-91	US-A-	5110904	05-05-92
		AU-B-	5789294	26-05-94
		AU-A-	6028690	07-02-91
		CA-A-	2022444	08-02-91
		JP-A-	3101695	26-04-91
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